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Mouse hepatitis virus A59-induced demyelination can occur in the absence of CD8⁺ T cells

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Mouse hepatitis virus causes a chronic demyelinating disease in C57BL/6 mice. While early studies suggested demyelination is due to direct cytolytic effects of virus on oligodendrocytes, there is increasing evidence for the involvement of the immune system in the mechanism of demyelination. In this study we have asked whether demyelination can occur in the absence of functional MHC class I expression and CD8⁺ T cells. We infected transgenic mice lacking expression of β_2 microglobulin ($\beta_2M^{-/-}$ mice) with MHV-A59. In $\beta_2M^{-/-}$ mice, virus was much more lethal than in either of the parental strains used to produce the mice; furthermore, while clearance from the CNS did occur in $\beta_2M^{-/-}$ mice, it was slower than in C57BL/6 mice. This is consistent with the importance of CD8⁺ cells in viral clearance. Because of the increased sensitivity of the $\beta_2M^{-/-}$ mice to infection, only low levels of virus could be used to evaluate chronic disease. Even at these low levels, demyelination did occur in some animals. To compare infection in $\beta_2M^{-/-}$ and C57BL/6 mice we used a higher dose of an attenuated variant of MHV-A59, C12. The attenuated variant induced less demyelination in C57BL/6 mice compared to wild type A59, but the levels observed were not significantly different from those seen in $\beta_2M^{-/-}$ mice. Thus, MHV-induced demyelination can occur in some animals in the absence of MHC class I and CD8⁺ cells.

Introduction

The coronaviruses are common pathogens of many animals. Mice infected experimentally with various strains of mouse hepatitis virus (MHV), most notably A59 or JHM, have been studied as a model of viral infection of the central nervous system (CNS). In this model, the virus replicates in glia (both astrocytes and oligodendrocytes^{1,2}) with detectable viral RNA and antigen in neurons^{3–4} and causes an encephalomyelitis. Results of some studies have suggested that neurons may provide a means of viral transport within the CNS^{3,4}. Clearance of virus occurs within 2–

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3 weeks postinfection (pi) and requires both CD8⁺ and CD4⁺ T cells^{6,7}. The CD8⁺ cells were suspected of acting as cytotoxic effector cells, and recent data demonstrating that CD8⁺ cytotoxic T cells specific for viral proteins are present in the CNS of mice infected with JHM suggests that this is indeed the case⁸.

Although infectious virus is recovered only rarely from animals after resolution of the acute disease, a few reports have demonstrated virus or viral antigen or RNA persisting in mice months after infection^{9–11}. During this time, a subacute disease develops that is characterized by inflammation and primary demyelination of neuronal axons in the CNS.

While the mechanism involved in viral clearance and the resolution of the acute-phase disease is becoming clearer, the factors important in the onset of demyelination are poorly understood. Early experiments demonstrated by electron microscopy the presence of virions in oligodendrocytes and led to the suggestion that viral cytolysis of oligodendrocytes was responsible for demyelination^{12,13}. There is now substantial evidence suggesting participation of the immune system in the pathogenesis of chronic demyelination. Wang *et al.*¹⁴ have shown that irradiation of JHM-infected mice prevents demyelination and that the transfer of T cells from immune mice can partially restore disease. Furthermore, JHM infection of rats results in an autoimmune reaction against myelin basic protein¹⁵ (MBP) and JHM infection of mice leads to autoreactive T cells of unknown specificity¹⁶. Both CD4⁺ and CD8⁺ T cells are localized to foci of JHM-induced demyelination and appear to be activated (S. Perlman, personal communication). Zimprich *et al.*¹⁷ also demonstrated CD4⁺ and CD8⁺ T cells in the demyelinated lesions induced by JHM in rats. Enhanced expression of MHC RNA or antigens in CNS-derived cells has been reported in both A59^{18,19} and JHM²⁰ infections. The induction of MHC antigens on glial cells may be part of an immune-mediated process that leads to demyelination.

We report here experiments aimed at determining the role of cellular immune responses, specifically CD8⁺ T cells, in MHV-induced demyelination. Our approach used mice carrying a deletion in the β_2 -microglobulin (β_2 M) gene which prevents the expression of functional MHC class I molecules and the maturation of CD8⁺ T cells. Consequently, these β_2 M^{-/-} mice are more susceptible to virus infection and do not clear virus as efficiently as normal C57BL/6 mice. However, demyelination in the brain and spinal cord occurs in some mice despite the absence of CD8⁺ cells. These results clearly demonstrate that CD8⁺ cells are not absolutely necessary for demyelination.

Results

Initial experiments showed that β_2 M^{-/-} mice were more susceptible to MHV-A59 following intracerebral (ic) inoculation of virus than were C57BL/6 (B6) mice. Infection

Table 1 Log₁₀ PFU per LD₅₀ of wild type A59 and C12

Virus	Mouse strain		
	C57BL/6	129/J	β_2 M ^{-/-}
Wild type	3.7	>5	0.7
C12	6.0	ND ^a	2.0

^a Not done.

of $\beta_2M^{-/-}$ mice with 5 000 plaque forming units (PFU) of A59 (one LD₅₀ for B6 mice) reproducibly killed $\beta_2M^{-/-}$ mice within 10–14 days. Consequently, we determined the LD₅₀ of A59 in $\beta_2M^{-/-}$ mice as well as in both parental strains, B6 and 129.²¹ As shown in Table 1, the LD₅₀ of A59 in $\beta_2M^{-/-}$ mice is 10³- to 10⁴-fold lower than in either $\beta_2M^{+/+}$ parent. In addition, we determined the LD₅₀ for C12, a fusion defective and attenuated mutant of A59.^{22,23} The LD₅₀ of C12 in B6 mice was previously determined to be 10⁶ pfu.²³ Although C12 retained its attenuated phenotype, the LD₅₀ for C12 in $\beta_2M^{-/-}$ mice was 10⁴-fold less than in B6 mice. The lower pfu per LD₅₀ observed for A59 in $\beta_2M^{-/-}$ mice compared to either parental mouse strain shows that the susceptibility of $\beta_2M^{-/-}$ mice is unique and is not a trait derived from either parent.

CD8⁺ T cells were shown previously to play an important role in the clearance of MHV-JHM from the CNS of B6 mice.^{6,7} The absence of a detectable CD8⁺ T cell population in naive $\beta_2M^{-/-}$ mice led us to examine the possibility that A59 would not be cleared efficiently and that a persistent infection of the CNS might result. For these experiments, $\beta_2M^{-/-}$ mice were inoculated ic with 5–20 pfu of wild type A59. (Three different amounts of virus were used for inoculation to ensure that we would have enough animals to measure replication of virus. We observed that 5 pfu (1 LD₅₀) did not reliably result in virus replication in all animals inoculated and that 20 pfu (4 LD₅₀s) resulted in a significant amount of mortality.) At selected times postinfection (pi) mice were sacrificed, perfused with PBS, and the brain, spinal cord, and liver removed. The tissue was homogenized and assayed for infectious virus. During the first 7 days pi, titers in the brain rose rapidly, achieving peak titers of 10⁸ pfu/g (Fig. 1A). Clinical signs of disease usually appeared at this time and were evidenced by ruffled fur and ataxia. Levels of virus remained relatively stable between 7 and 14 days pi with little evidence of clearance until day 15. During this time the mice developed increasingly severe disease which often presented as hind limb paralysis; most deaths occurred during the second and third week pi. Infection of B6 controls with 5000 pfu (approx. 1 LD₅₀) also led to a rapid increase in virus titer during the first week, but in contrast to infection of $\beta_2M^{-/-}$ mice, virus clearance was essentially complete by 2 weeks pi (Fig. 1A). Similar patterns of virus growth in the liver of B6 and $\beta_2M^{-/-}$ mice were observed with transient growth followed by clearance in B6 mice but growth to high titer and delayed clearance in $\beta_2M^{-/-}$ mice (data not shown).

The replication of virus to significantly higher titer together with the results suggesting poor viral clearance in $\beta_2M^{-/-}$ mice meant that any demyelination observed later could be due directly to the lytic effect of virus persisting in the CNS. Consequently, brains and spinal cords of mice killed at 30 days post-infection (pi) or later were processed for both titration of infectious virus and for histopathology. Animals were perfused with PBS and the brain and spinal cords were removed and divided in half. One half of each tissue was fixed in buffered formalin and used for histopathologic examination whereas the remaining half was homogenized and used for titrations. Titration of these samples showed that with two exceptions, virus was cleared from the brains by 30 days pi (Fig. 1B). One animal killed at 60 days pi without obvious physical signs of disease had 5×10^4 pfu per g of tissue. The other animal was killed at 76 days pi with severe hind limb paralysis and had a similar virus titer in the brain. Despite these two exceptions, clearance of virus from the CNS of $\beta_2M^{-/-}$ mice was generally complete by 30 days pi.

Sections of brain and spinal cord from these same $\beta_2M^{-/-}$ mice were stained with luxol fast blue (LFB) to identify regions of myelin loss. Since MHV-induced demyelination occurs with preservation of axons (i.e. is primary demyelination) LFB is a reliable marker for myelin loss. As shown in Table 2, only three of 58 (5.2%) mice

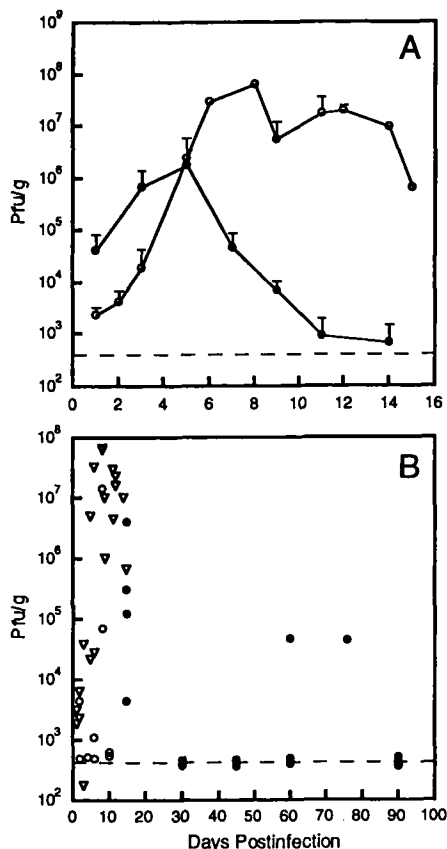


Fig. 1. Viral growth kinetics and clearance. (A) $\beta_2M^{-/-}$ (\circ) and B6 mice (\bullet) were inoculated ic with 20 pfu and 5000 pfu, respectively, of A59. At the indicated times, two mice were killed without regard to signs of disease and their brains removed. Tissue was homogenized and infectious virus was assayed as described in Materials and Methods. Plotted are the mean and standard deviation for each pair of mice except for $\beta_2M^{-/-}$ mice sacrificed on days 14 and 15 in which data were obtained from a single mouse. The limit of detection in this assay is indicated by the dotted horizontal line. (B) $\beta_2M^{-/-}$ mice were inoculated ic with 5 (\circ), 10 (\bullet), or 20 (∇) pfu of A59. At the indicated times, virus present in the brain was determined as in Fig. 1A. (All animals inoculated with 20 pfu were sacrificed by 15 dpi). Each symbol represents one animal. The limit of detection in this assay is indicated by the horizontal line. Larger amounts of lysate were assayed in one sample resulting in a lower level of detection; this is indicated by the one triangle located below the dotted line.

examined between 15 and 90 days pi had demyelinating lesions in their brain or spinal cord.

The demyelinating lesions in both $\beta_2M^{-/-}$ and B6 mice consisted of focal areas of mild to moderate meningeal and perivascular lymphocytic infiltration and scattered inflammatory infiltration within the white matter parenchyma of the spinal cord and brain. Vacuolization with edema of the neuropil and foamy macrophages were also present. These inflammatory white matter lesions exhibited reduced LFB staining without evidence of infarction or axonal damage, indicative of a primary demyelinating process (Fig. 2). When demyelination was scored as the percent of spinal cord quadrants affected, only 18 (2.2%) of the 812 quadrants were found to contain lesions. The extent of demyelination did not appear to change over time with the possible exception of the group of mice killed at 60 days pi which was 2–5 times higher than at earlier times. This higher level of demyelination was due to a single

Table 2 Pathology in $\beta_2M^{-/-}$ mice infected with wild type A59 or C12

Virus ^a	DPI	N ^b	Normal	Encephalitis alone	Inflammatory demyelination	Demyelination		Virus Detected ^d
						Score ^c	(%)	
Wt	15	4	1	3	0	0/44	(0.0)	3/4
	30	14	7	6	1	3/236	(1.3)	0/6 ^e
	45	14	11	2	1	4/144	(2.8)	0/14
	60	11	10	0	1	11/180	(6.1)	1/6 ^f
	90	15	11	4	0	0/208	(0.0)	0/15
	Total	58	40	15	3	18/812	(2.2)	4/45
C12	30	11	3	4	4	4/132	(3.0)	1/8 ^g
	45	8	4	0	4	5/96	(5.2)	0/7
	60	12	8	2	2	6/140	(4.3)	0/12
	Total	31	15	6	10	15/368	(4.1)	1/27

^a Mice were infected ic with 5–10 pfu of Wt or 200 pfu of C12.

^b Number of mice examined.

^c Number of spinal cord quadrants exhibiting demyelination/number of quadrants examined.

^d Number of mice containing infectious virus/number examined.

^e Animal with demyelination unavailable for titration.

^f Demyelinated animal virus positive.

^g Single virus-positive animal did not exhibit demyelination.

mouse with extensive lesions: 11 of 12 spinal cord quadrants examined were affected. Interestingly, this animal was one of the two still harboring infectious virus at the time of sacrifice. Of 31 mock-infected control mice examined between 15 and 90 days pi, none exhibited demyelination in either the brain or the spinal cord.

Because of the difficulties in establishing an infection in every inoculated animal when using low doses of virus, we also included experiments with the C12 variant of MHV-A59²³ which, being attenuated, allowed us to use larger amounts of virus. In these experiments, mice were infected ic with 200 pfu of C12 and then killed between 30 and 60 days pi. Pathologic examinations were done as described above for wild type-infected $\beta_2M^{-/-}$ mice. The percent of animals infected with C12 and exhibiting lesions of demyelination varied from 16.7% to 50% (2 of 12 and 4 of 8, respectively). However, the percentage of total quadrants of spinal cord affected was only 4.1%, and the prevalence of demyelination was not dependent on the time of sacrifice of the animals. Twenty-five mock-infected controls sacrificed at the same times as the experimental group did not show any signs of disease or demyelination.

These results clearly show that demyelination can occur despite the absence of CD8⁺ T cells in infected mice. To determine if the lack of CD8⁺ cells altered the occurrence or severity of demyelination, we infected B6 mice with 200 pfu of either wild type A59 or C12 and prepared sections of brain and spinal cord 35 days pi. In wild type infected B6 mice, four of seven (57.1%) mice showed lesions with 27 of 144 (18.8%) quadrants positive (Table 3). This is in marked contrast to C12-infected B6 mice. Here, only one of seven (14%) mice exhibited demyelination, and in this one animal, only one lesion was observed among 144 quadrants examined. Therefore, the attenuated phenotype of C12 (Table 1) is also reflected in the severity of demyelination in ic-infected B6 mice. Inoculation of the same dose of C12 into $\beta_2M^{-/-}$ mice induced demyelination in four of 11 animals, and examination of LFB-stained sections revealed lesions in 3% (4 of 132) quadrants. This frequency is not significantly different from that observed in B6 mice ($p > 0.05$ by the Mann–Whitney U test) and argues that the absence of CD8⁺ T cells does not result in a reduced levels of demyelination.

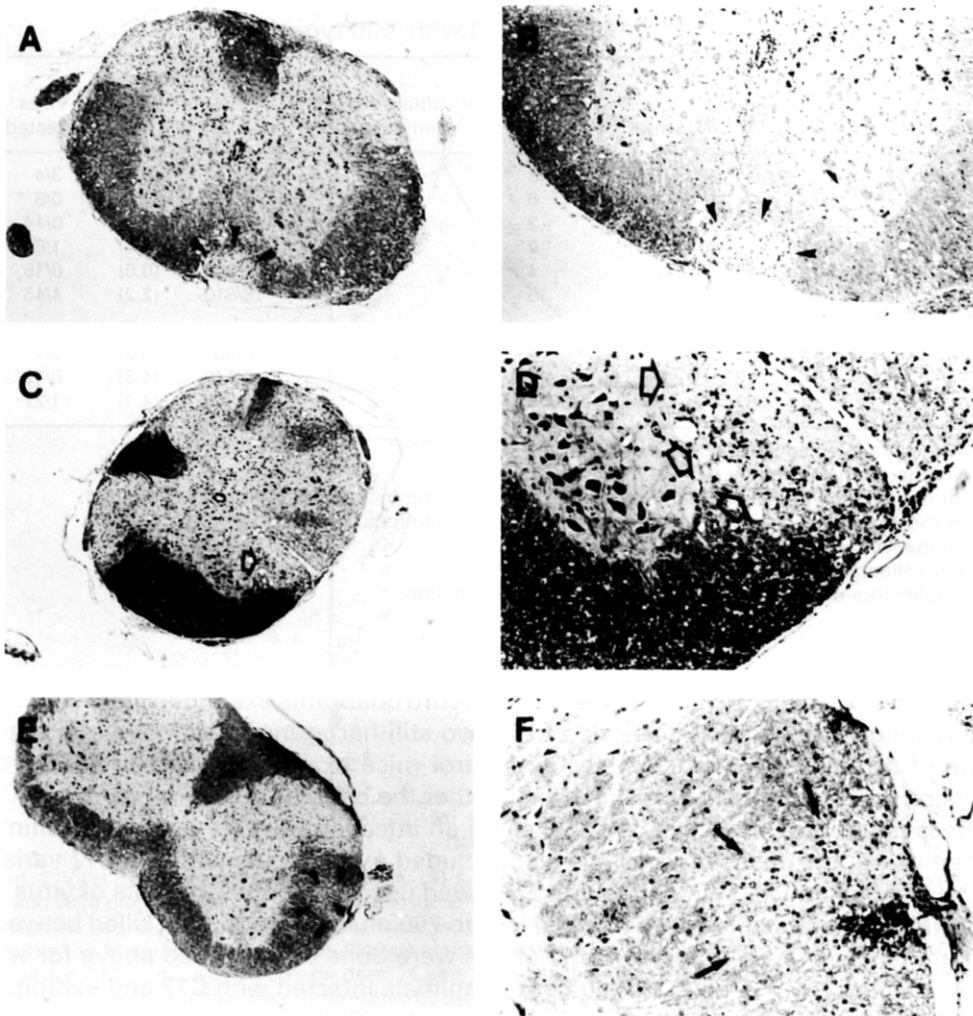


Figure 2. MHV induced demyelination in $\beta_2M^{-/-}$ and B6 mice. Spinal cord sections from mice killed from 36–60 days after inoculation. All sections were formalin fixed, paraffin embedded and stained with luxol fast blue. White matter demyelinating lesions are indicated by arrows or arrowheads. Panels A and B. Sections from a $\beta_2M^{-/-}$ mouse inoculated with MHV-A59. Panel A shows a cross section of the entire spinal cord exhibiting focal mononuclear inflammatory infiltrate in the meninges adjacent to the anterior and posterior midline. In addition, a demyelinating lesion is observed in the anterior column of the white matter ($\times 40$). Panel B shows a higher magnification of the area of demyelination ($\times 100$). Panels C and D. Sections from a B6 mouse inoculated with MHV-A59. Panel C shows a cross section of the entire spinal cord showing focal meningitis and extensive demyelination in both anterior aspects of the cord and one of the lateral columns. A sharp border between the demyelinating lesion and the intact myelin is shown by the arrows. Note that the border between gray and white matter becomes obscured in the demyelinated areas. Panel D shows a higher magnification of an area of demyelination and inflammation ($\times 200$). Panels E and F. Sections from a $\beta_2M^{-/-}$ mouse inoculated with the C12 variant of MHV-A59. Panel E shows a cross-section of the spinal cord showing focal meningitis; an area of demyelination and inflammation in the lateral aspect of the cord is indicated by the arrows ($\times 40$). Panel F shows a higher magnification of the area of demyelination and inflammation ($\times 200$). Reproduced here at 70%.

Discussion

MHV infection of the CNS of immunocompetent mice causes an acute encephalomyelitis that will last 2–3 weeks.^{3,24} During this time, virus replicates and is subsequently cleared in a CD4⁺- and CD8⁺-T cell dependent manner.^{6,7,25} Histological examination of tissue shows primary demyelination of neuronal axons in the brain

Table 3. Comparison of the pathology in B6 and $\beta_2M^{-/-}$ mice

Virus ^a	Mouse Strain	DPI	N ^b	Normal	Encephalitis alone	Inflammatory demyelination	Demyelination	
							Score ^c	(%)
Wt	B6	35	7	1	2	4	27/144	(18.8)
C12	B6	35	7	0	6	1	1/144	(0.7)
	$\beta_2M^{-/-}$	30	11	3	4	4	4/132	(3.0)
Mock	B6	35	6	6	0	0	0/108	(0.0)
	$\beta_2M^{-/-}$	30	9	9	0	0	0/108	(0.0)

^a Mice were infected ic with 200 pfu of Wt or C12.

^b Number of mice examined.

^c Number of spinal cord quadrants exhibiting demyelination/number of quadrants examined.

and spinal cord. Initial studies showing that immunosuppression did not prevent demyelination and the presence of coronavirus-like particles in oligodendrocytes of infected mice led to the suggestion that demyelination was the result of oligodendrocyte degeneration.^{12,13} Subacute or chronic demyelination is observed at later times after infection (1–12 months) when infectious virus is usually not detectable, which implies that virus-induced degeneration of oligodendrocytes is not the sole mechanism of demyelination. Watanabe *et al.*¹⁵ reported the adoptive transfer of subacute demyelination with lymphocytes from infected syngeneic rats. More recent experiments support a role for an immunopathological mechanism by showing that irradiation of mice within the first week of infection prevented demyelination.^{14,26} Demyelination could be partially restored with the adoptive transfer of T cells from immune donors. While the evidence for an immune-mediated mechanism in MHV-induced demyelination is strong, it is important to consider that both mechanisms may contribute to disease. Indeed, it has been suggested that direct viral cytolysis/cytotoxicity may be operational during the early phase (7–10 days) of disease and an immune-mediated component (with or without viral involvement) in the later subacute phase.²⁴

In the experiments reported here, we took advantage of the availability of $\beta_2M^{-/-}$ mice²¹ to investigate the role CD8⁺ T cells play in MHV-induced subacute demyelination. If CD8⁺ T cells are significant in the induction of demyelination, the subacute disease might be expected to decrease substantially. In contrast, if the pathway to demyelination does not involve CD8⁺ cells, but is due to either another immune cell or to oligodendrocyte degeneration, the frequency of demyelination should be unaffected or possibly enhanced since CD8⁺ cells are required for viral clearance from the CNS and virus replicates to higher levels in their absence (Fig. 1A).

When compared to previous studies of A59-infected B6 mice,^{3,25} there is little difference in the course of infection except that in $\beta_2M^{-/-}$ mice the virus grows to higher titer and is cleared more slowly. Indeed, the delayed clearance of virus was expected and supports the role of CD8⁺ cells in recovery from the acute disease. A similar defect in the clearance of sendai virus from $\beta_2M^{-/-}$ mice has been reported.²⁷ However, the results of Eichelberger *et al.*²⁸ showing normal clearance of influenza virus in $\beta_2M^{-/-}$ mice demonstrate that the loss of CD8⁺ T cells and MHC class I antigens do not necessarily lead to defects in viral clearance. While the means by which A59 is cleared in the $\beta_2M^{-/-}$ mice is unclear, the results of Muller *et al.*²⁹ suggest MHC class II-restricted CD4⁺ cytotoxic T cells are one candidate. Measurement of antibody responses in $\beta_2M^{-/-}$ mice following infection with either Theiler virus^{30,31} or influenza virus²⁸ show that neutralizing antibody levels are similar to those in

$\beta_2M^{+/+}$ mice, indicating that humoral responses could also play a role in the clearance of A59 from these mice.

Pathologic examination of the brain and spinal cord of $\beta_2M^{-/-}$ mice taken at late times pi (1–3 months) revealed clearly discernible demyelinating lesions. Inflammatory demyelination did not correlate with the presence of virus in either tissue. Indeed, most mice exhibiting demyelination did not contain detectable levels of virus. Over all time periods, we found 3 of 58 (5%) wild type-infected mice and 10 of 31 (33%) C12-infected mice contained demyelinating lesions. The ability to induce demyelination in $\beta_2M^{-/-}$ mice points out the absence of an absolute requirement for CD8⁺ T cells in this disease. Since virus was rarely detectable in the CNS of demyelinated animals, we suggest that the pathology that was observed arose by a mechanism that does not require ongoing infection or CD8⁺ cells.

CD8⁺ T cells are also not required for Theiler's virus-induced demyelination. Infection of $\beta_2M^{-/-}$ mice with Theiler's virus results in delayed viral clearance and demyelination.^{30, 31} Interestingly, H-2^b haplotype mice are usually resistant to Theiler's virus-induced disease; in the absence of CD8⁺ T cells, however, clearance of virus is delayed and demyelination does occur. A difficult question to answer is whether MHV-induced demyelination in B6 mice is more extensive or severe than in $\beta_2M^{-/-}$ mice. Previous studies have shown an inherent inefficiency in successfully infecting mice with low doses of virus (Gombold and Sutherland, unpublished data). Therefore, the 5% demyelination observed in wild type-infected $\beta_2M^{-/-}$ mice can only be taken as a lower limit of the actual frequency. It was for this reason that C12 was used in the second group of experiments. C12 is attenuated in both B6²³ and $\beta_2M^{-/-}$ mice (Table 1) which allowed us to use higher doses of virus. The attenuation of C12 may be reflected in the extent of demyelination seen in infected B6 mice; compared to wild type virus at the same dose (200 pfu), C12 caused 25 times less demyelination (18.8% vs 0.7%). However, the inoculation of the same dose of C12 into $\beta_2M^{-/-}$ mice resulted in levels of demyelination that are not statistically different from C12-infected B6 mice. Therefore, neither the loss of CD8⁺ T cells nor the elevated titers of virus that occur in $\beta_2M^{-/-}$ mice lead to a change in the level of demyelination. We are cautious about extending this conclusion to wild type virus, however, because the levels of demyelination may be less with C12 than with wild type A59.

A major concern with these experiments was ensuring that our colony of $\beta_2M^{-/-}$ mice were truly deficient for CD8⁺ cells. Routine analyses of cells from the spleen and lymph nodes from naive $\beta_2M^{-/-}$ animals failed to detect expression of β_2M or CD8⁺ cells (data not shown), in agreement with the original observations on these mice.²¹ It has been demonstrated recently, however, that $\beta_2M^{-/-}$ mice are competent to mount a CD8⁺ response against allogeneic tumor cells after *in vivo* priming.^{32–34} Presumably, therefore, the low level of expression of MHC class I heavy chain on the surface of the cell in the absence of β_2M ³⁵ is sufficient to select for low numbers of CD8⁺ cells which recognize such ligands after suitable *in vivo* expansion. It should be noted that the response to allogeneic MHC molecules usually occurs at very high frequency in normal naive mice; 0.3 to 1% of peripheral T cells will respond to alloantigen³⁶ in the absence of *in vivo* expansion. Nevertheless the presence of CD8⁺ T cells in $\beta_2M^{-/-}$ mice, which can be expanded by *in vivo* manipulation, raises the possibility that such cells may be recruited during the acute or chronic phase of MHV infection. This would require, however, the presentation of antigenic peptides by MHC class I heavy chain alone, which has been shown not to occur except in the presence of β_2M .^{37–39} In contrast, alloresponses are thought to be mediated by a wide variety of ligands which include a large set of endogenously and exogenously derived peptides in association with the allogeneic MHC, in addition to the MHC molecule

itself.⁴⁰ It is not surprising therefore that the low number of CD8⁺ T cells in $\beta_2M^{-/-}$ mice can find a suitable stimulus within this population of ligands. We believe, therefore, that it is highly unlikely that CD8⁺ T cells competent to respond to MHV are present in these animals and this belief is supported by our finding that these animals are almost incapable of mounting a protective immune response against the virus.

Materials and methods

Virus and Cells. MHV strain A59 and C12, the attenuated mutant derived from it, were described previously.^{22,23} These viruses were grown routinely in L2 cells using DMEM supplemented with 10% fetal bovine serum. The titration of infectious virus in homogenized tissue samples was performed at 37 °C as described previously.²²

Mice. $\beta_2M^{-/-}$ mice were obtained originally from Dr. Beverly Koller (Univ. of North Carolina, Chapel Hill USA) and have been described previously.²¹ Breeding pairs were housed in micro-isolator cages in the Univ. Pennsylvania animal facilities. Four- to six-week-old male, MHV-antibody free, C57BL/6 mice and 129/J mice were purchased from Jackson Laboratories (Bar Harbor, ME USA), and housed in the same manner as $\beta_2M^{-/-}$ mice.

Inoculation of mice. We diluted A59 or C12 with phosphate-buffered saline (PBS) containing 0.75% bovine serum albumin. Mice were anesthetized with Metofane (Pittman-Moore, Mundelein, IL USA) and 20 μ l of diluted virus was injected into the left cerebral hemisphere. Mock-infected controls were inoculated similarly but with an uninfected cell lysate at a comparable dilution. Virus-infected animals and controls were housed in isolation for the duration of the experiments.

LD₅₀ assays were performed as described previously.²³ Briefly, groups of 5–10 mice were inoculated with serial dilutions of wild type A59 as described above. Mice were examined for signs of disease or death on a daily basis up to 21 days pi. LD₅₀s were calculated by the Reed-Meunch method. The LD₅₀ of C12 in $\beta_2M^{-/-}$ mice was determined similarly except that fewer mice and virus dilutions were used.

Histology. At selected times postinfection, mice were anesthetized, perfused with PBS, and brains, spinal cords, and livers removed. Brains were cut sagittally at the midline, and one half was placed into gelatine saline,⁴¹ weighed, and stored at –70 °C for virus titrations; the remaining half was fixed overnight in phosphate-buffered formalin. Spinal cords were cut into six 3–5 mm pieces. One half of the cord (using alternating pieces) was placed into gelatine saline, weighed, and stored at –70 °C; the remaining pieces were fixed in formalin. Livers were placed into gelatin saline, weighed, and stored at –70 °C.

Formalin-fixed tissue was embedded in paraffin, sectioned, and stained with Hematoxylin and Eosin (H&E) or with Luxol Fast Blue (LFB). H&E-stained sections were used for pathologic evaluation while LFB-stained sections were examined to identify regions of demyelination. The extent of demyelination was calculated as the percent of spinal cord quadrants containing lesions. Pathologic examinations were done blind using coded samples.

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